AD_____

Award Number: DAMD17-01-1-0340

TITLE: Epstein-Barr Virus and Breast Cancer

PRINCIPAL INVESTIGATOR: Wing C. Chan, M.D.
Kishor G. Bhatia, Ph.D.
Guimei Zhou

CONTRACTING ORGANIZATION: University of Nebraska Medical Center
Omaha, Nebraska 68198-6810

REPORT DATE: September 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2002	Annual (1 Sep 01 - 31 Aug 02)

4. TITLE AND SUBTITLE
Epstein-Barr Virus and Breast Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0340

6. AUTHOR(S)
Wing C. Chan, M.D.
Kishor G. Bhatia, Ph.D.
Guimei Zhou

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Nebraska Medical Center
Omaha, Nebraska 68198-6810

E-Mail: jchan@unmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Epstein Barr virus (EBV) has been implicated as a cofactor in several human malignancies. The possibility that EBV may play a role in the development of breast cancer has been raised in recent years. However, a number of reports have shown conflicting results. This could be related to the different assays employed and also possible geographical variations in the incidence of this infection. We collected 203 cases of invasive breast carcinomas, as well as 30 non-neoplastic tissues adjacent to the tumors from 3 different geographical regions (USA, India and Kuwait), conventional polymerase chain reaction (PCR), real-time PCR and EBV-encoded small nonpolyadenylated RNA (EBER-1) in situ hybridization (ISH) were used to study these cases. The data suggest that EBV is present in a small subset of breast carcinomas, however, the high PCR positivity is likely due to the presence of latently infected lymphocytes; the incidence and/or the viral load of EBV associated with breast cancers may be geographically variable and there is a discrepancy between the PCR and ISH assays. Whether the discrepancy is only due to the detection of EBV by PCR on infiltrated lymphocytes or there is deleted EBV in tumor cells that fail to express EBERS needs to be explored by further studies.

14. SUBJECT TERMS
breast cancer, Epstein-Barr virus (EBV), pathogenesis

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

EBV is a γ-herpesvirus and infects more than 90% of the world population (1). The virus normally maintains a latent infection without symptoms, however, in the presence of impaired immunity or with additional genetic changes in virus-infected cells, EBV may be an important cofactor in the development of several human malignancies. Malignancies that have strong evidence for EBV include African Burkitt's lymphoma (2), nasopharyngeal carcinoma (NPC) (3), classical Hodgkin's disease (4), post-transplant and AIDS-associated lymphomas (5,6), nasal NK/T cell lymphoma (7), and gastric carcinomas (8). These are diseases that reflect the predominant tropism of EBV for two distinct cell types: B- lymphocytes and epithelial cells. A new study showed the entry of EBV to epithelial cells follows substantially different pathway compared to its entry to B-lymphocytes (9).

Breast cancer is the most frequent malignancy and the leading cause of cancer death among women in Western countries. Although the etiology of breast cancer is not completely understood, genetic background and hormonal effects are believed to play important roles in its development (10). With increasing reports of the association of EBV with epithelial cell malignancies, researchers have raised the question of whether EBV may play a role in the development of breast cancer. However, a number of reports on its association with breast cancer have shown conflicting results (11-18). This could be related to the different assays employed and also possible geographical variations in the incidence of this infection.

In an attempt to resolve this dispute, the aims of our study are: 1) To compare if different assays for the detection of EBV provide different endpoints. 2) To compare if there is a geographical variation in the association of EBV with breast cancer samples obtained from the USA, Middle Eastern countries, India and China. 3) To determine if the presence of EBV in these samples is a) restricted to tumor cells and b) if the virus was present prior to the clonal expansion of the tumor cells. 4) To assess the overall expression pattern of viral genes in EBV positive breast cancer specimens.

For the study of geographical variations, we collected 203 cases of primary invasive breast cancers from three different areas (USA, India and Kuwait, more cases from different geographic regions will be collected later). Different assays were used to assess the presence of EBV in these tumors, including conventional PCR, real-time PCR and EBER ISH. Immunohistochemistry and laser capture microdissection (LCM) techniques have been developed and will be employed for further studies.

BODY

We collected 100 cases of invasive breast carcinomas, as well as 30 non-neoplastic tissues adjacent to the tumors to study for evidence of Epstein-Barr virus (EBV) infection. DNA was extracted from the paraffin embedded tissues using EX-WAX DNA extraction kit (Intergen Company, 2 Manhattanville Road, Purchase, NY 10577) according to the manufacturer's instructions. Amplification of GAPDH or beta-globin gene was used to test the integrity of DNA for all breast cancer samples. Conventional PCR were performed on 30 cases including both tumor and benign tissues, using sets of primers flanking the EBV nuclear antigen (EBNA)-LP, latent membrane protein (LMP)-1, the transactivating immediate-early BZLF-1 and EBNA-3C genes. The rest of the samples were tested for the BZLF-1 region of EBV DNA only for the time been. Nineteen of 30 tumor tissues were positive for at least one set of primers (3 cases were positive for all), including 9 cases that were PCR positive for both tumor and benign tissues, see Figure 1. and Table 1.

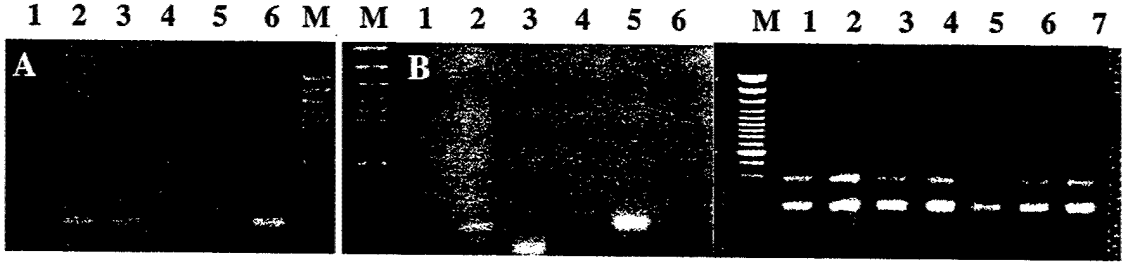

Figure 1. PCR amplification of BZLF-1 (A) and LMP-1 (B) genomic DNA on 4 breast cancer samples (lane 1-4 in both A and B). Lane M is a 100 base pair (bp) Lane 3 represents a sample which is PCR positive for BZLF-1, but negative for LMP-1. Lane 6 (A) and Lane 5 (B) are Namalwa DNA. Lane 5 (A) and Lane 6 (B) are no template controls. (C) Breast cancer samples amplified with human β-globin DNA to demonstrate good DNA quality.

Figure 2. EBER-1 in situ hybridization in positive control and breast cancer sections. (A) shows strong nuclear staining in a control with posttransplant lymphoproliferative disorder.(B) Case #20 shows nuclear positivity in the infiltrated lymphocytes. (C) Positive staining was detected in focal tumor cells (case # 17R).

However, the correlation among the primer sets used was poor. This may due to the very low copy number of EBV present in tissues and some tissues may have partially deleted EBV genome (19,20). Thus, real-time PCR was employed to quantitate the number of EBV genome in tissues. A106-bp region of EBV BamH1K encoding EBNA1 was amplified to detect wild type EBV genome. A 90-bp region that encompassed the junction of rearranged DNA was amplified to detect defective, rearranged EBV genome. A 72-bp DNA sequence of human ApoB gene was used as an internal control. Preliminary data showed that cases, which only have one set of primer positive for PCR, had lower load of the EBV genome. See Figure 3.

Table 1. Summary of EBV-positive breast cancer by PCR and EBER in situ hybridization

	Tumor					Benign				
Case no.	BZLF-1	BWRF-1	LMP-1	EBNA-3C	EBER ISH	BZLF-1	BWRF-1	LMP-1	EBNA-3C	EBER ISH
2	-	+	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	+	-	-
4	+	+	+	+	-	-	-	-	-	-
6	+	-	-	+	-	-	-	-	-	-
8	+	-	-	-	-	+	-	-	-	-
9	+	-	-	-	-	NA	NA	NA	NA	NA
12	+	+	-	-	+L	+	-	-	-	-
13	+	-	-	-	-	+	-	-	-	-
14	+	-	-	-	-	-	-	-	-	-
15	+	-	-	-	-	+	-	-	-	-
17R	+	+	-	-	+T	+	-	-	-	-
17L	-	-	-	-	-	+	-	-	-	-
18	+	-	-	-	-	-	-	-	-	-
19	+	-	-	-	-	-	-	-	-	-
20	+	+	-	+	+L	+	-	-	-	-
22	+	-	-	-	-	-	-	-	-	-
23	+	-	-	-	-	+	-	-	-	-
25	+	-	-	-	-	-	-	-	-	-
27	+	-	-	-	-	NA	NA	NA	NA	NA
29	+	-	-	-	-	-	-	-	-	-

NA- not available; +L – positive in lymphocytes; +T – positive in tumor cells

Association of EBV with breast cancers was also assessed by our collaborator, Dr. Bhatia at King Faisal Specialist Hospital, Saudi Arabia with 103 breast cancers from two different geographical regions

40 breast tumors from Kidwai Cancer Center, Bangalore, India and 63 samples from Kuwait Cancer Center. To determine the presence of EBV, amplification of the region flanking BZLF1 gene was carried out. Only 3 of 63 samples from Kuwait were positive for EBV, whereas 16/40 Breast cancer samples from India were EBV positive.

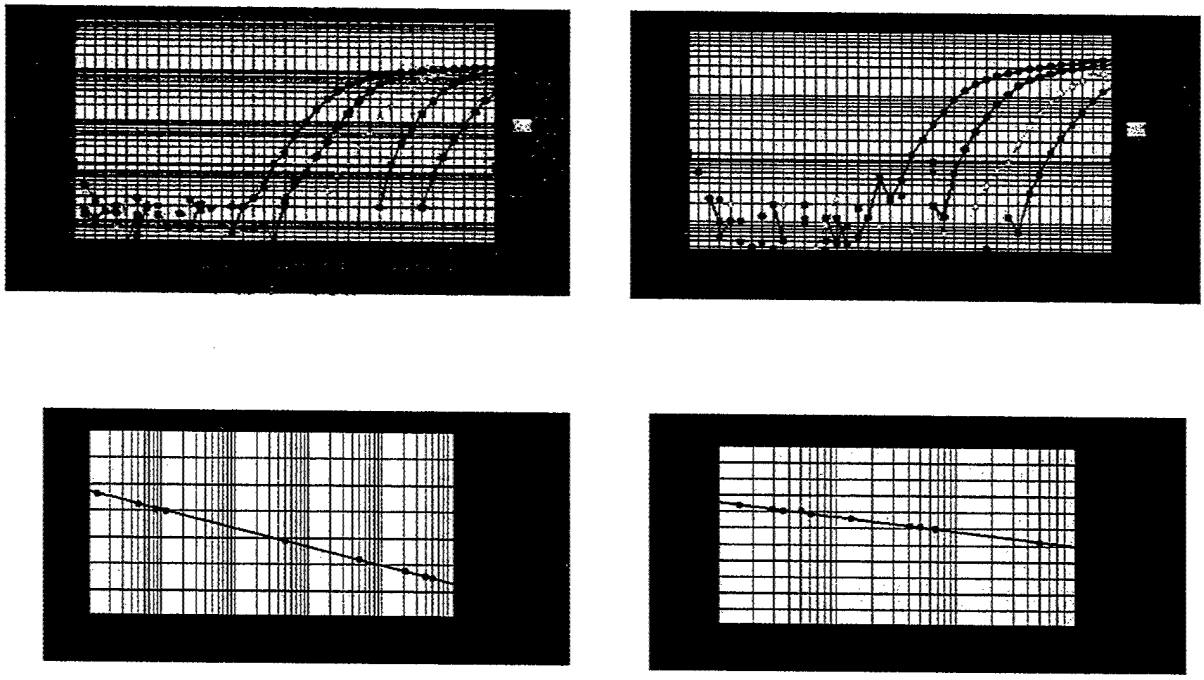

Figure3. Amplification plot of real-time PCR of EBNA-1. (A) Serially diluted plasmid containing EBV (100,000 to 10 copies per tube) were analyzed for controls. (B) Standard curve generated from the mean value of duplicated examinations. Black circles represent controls and red circles are breast cancer samples and cell line. The tested materials plotted are (from left to right) breast cancer case #2, #4 and Raji (an EBV positive lymphoma cell line). (C) and (D) Amplified for human Apo-B DNA to normalize DNA content of each sample. (C) Serially diluted RL-7 DNA (an EBV negative lymphoma cell line) from 1000 ng to 1 ng were analyzed for controls. (D) Same samples as tested in (B).

The 40 breast cancer biopsies from India and 30 samples form USA were also assessed for the expression of EBERs using in situ hybridization kit from DAKO (DAKO A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark). All, but 1 sample from the USA, including those that were PCR positive, failed to show expression of EBERs in tumor

cells. Two USA samples showed some staining of infiltrated lymphocytes. See Figure 2. and Table 2. These data suggest that the incidence and/or the viral load of EBV associated with breast cancers may be geographically variable and there is a discrepancy between the PCR and ISH assays. Part of the discrepancy may be due to the detection of EBV by PCR on infiltrating lymphoid cells. However, the possibility of the presence of partially deleted EBV in tumor cells that failed to express EBERs needs to be explored by further studies (21).

Table 2. PCR PRIMER AND PROBE SEQUENCES

BZLF-1	+5'-AGGCTGTGGAACACCAATG
	-5'-AACCCAGAATCAACAGACTAAC
BWRF-1	+5'-CCATGTAAGCCTGCCTCGAG
	-5'-GCCTTAGATCTGGCTCTTTG
LMP-1	+5'-CGGAAGAGGTTGAAAACAAA
	-5'-GTGGGGGTCGTCATCATCTC
EBNA-3C	+5'-AGAAGGGGAGCGTGTGTTGT
	-5'-GGCTCGTTTTTGACGTCGGC
EBNA-1	+5'-CCGGTGTGTTCGTATATGGAG
	-5'-GGGAGACGACTCAATGGTGTA
EBNA PROBE	FAM-TGCCCTTGCTATTCCACAATGTCGTCTT-TAMRA
APO-B	+5'-TGAAGGTGGAGGACATTCCTCTA
	-5'-CTGGAATTGCGATTTCTGGTAA
APO-B PROBE	VIC-CGAGAATCACCCTGCCAGACTTCCGT-TAMRA

In conclusion, our study showed that using PCR technique alone to detect the presence of EBV genome in breast cancer can obtain 40-60% positive cases. However, PCR cannot distinguish between neoplastic cells and latently infected lymphocytes. The high rates of positivity may indicate a lack of specificity. In an attempt to define the cellular localization of EBV in breast cancer, the morphology-based assay RNA ISH to detect the expression of EBER was employed. Very few cases turn out to be positive by this assay and in some positive cases, the reactive cells are lymphocytes rather than tumor cells. In contrast to conventional PCR, the use of Real-Time PCR has a number of advantages including improved specificity, decreased contamination and quantitative results. We have developed this assay and it has been applied to a few cases.

KEY RESEARCH ACCOMPLISHMENTS

- Established PCR assays for 6 EBV genes including one for defective viruses.

- Established EBER in situ hybridization for localization of EBV infected cells.

- Assayed 100 of USA cases, 63 of Kuwaiti cases and 40 of Indian cases of breast carcinoma for EBV.

- Developed Real-Time PCR assay for the EBNA1 region and also an assay for the defective EBV.

- Organized a data base for the experimental findings .

REPORTBLE OUTCOMES:

The study from this period generated an abstract titled " Epstein-Barr virus and breast cancer" and presented in the " Era of Hope – Department of Defense Breast Cancer Research Program meeting, 2002". See appendices

CONCLUSIONS:

We tested 203 primary invasive breast cancers as well as 30 non-neoplastic tissues adjacent to the tumors from 3 different geographical regions by amplifying EBV DNA region flanking the BZLF-1 gene, 30 cases from the USA which have both tumor and benign tissues were also tested for 3 other regions of EBV genome by PCR (see table 2 for primer sequences). These conventional PCR data showed that using BZLF-1 alone, we could detect EBV positivity in 5% of 63 fresh frozen tissues from Kuwait, 40% and 55% of paraffin embedded tissues from India and USA, respectively. 30 USA cases tested by PCR on 4 different EBV regions showed 63% positivity in tumors and 30 % in benign tissues. However, the correlation among the primer sets used was poor (figure 1 and 2). This maybe due to the very low copy number of EBV present in the tissues and some of the tissues may have partially deleted EBV genome (19,20). Sixbey JW and his co-workers reported that they successfully amplified sequences that span abnormally juxtaposed BamH1 W and Z fragments characteristic of defective heterogeneous (het) EBV DNA from 31% and 33% of EBER-positive and negative Hodgkin's disease, respectively. This may be true in breast cancers.

The EBER ISH was performed on 70 paraffin embedded sections from India and USA. All, but 1 sample from the USA, including those that were PCR positive, failed to show expression of EBERs in tumor cells. Two USA samples showed some staining of infiltrating lymphocytes. See Figure 2. and Table 2. These data suggest that the incidence of and/or the viral load of EBV associated with breast cancers may be geographically variable and there is a discrepancy between the PCR and ISH assays. Part of the discrepancy may be due to the detection of EBV by PCR on infiltrating lymphoid cells. However, the possibility of the presence of partially deleted EBV in tumor cells that failed to express EBERs needs to be explored by further studies (21).

These data suggest the incident of EBV associated with breast cancer is higher in the USA than the other two regions. This finding needs to be confirmed for a firm conclusion; however, some literature reported breast cancer risk is associated with "delayed" primary Epstein-bar virus infection (24), which is more frequent in developed countries.

These data also suggest that the high positivity of EBV in breast cancer by conventional PCR may lack specificity.

We will explore the role of Real-Time PCR in resolving some of these uncertainties in the future. In contrast to conventional PCR, Real-Time PCR should improve specificity by using a sequence-specific probe in the reporting system (22). Since the PCR is in a

closed system, it will avoid cross contamination which is a major concern in experiments involving amplification of viral genomes. We can also obtain the copy numbers of EBV genome present in the sample and hence have a good estimate of the level of infection present. We have developed this technique and have applied it to a few cases.

In an attempt to further define the cellular localization of EBV in breast cancer, morphology-based assays such as DNA in situ to detect EBV genome in addition to immunohistochemistry to assess the expression of latent protein LMPs and/or EBNAs will be established and applied to the study.

Laser capture microdissection [LCM] may also be used in this study (23). When combined with real–time PCR technique, it should provide a reliable mean of assessing the distribution of EBV in breast cancer. Our laboratory is experienced in the use of LCM and would not anticipate any technical problems in using it for this project.

REFERENCES

1. Mandell, G.L. Douglas, R.G., and Bennett, J.E. 1990. Principles and Practice in Infectious Diseases, Ed. 3. New York: Churchill Livingstone.
2. Burkitt's Lymphoma. Semin Cancer Biol. Oct: 3(5): 285-95
3. Crunchley, A.T., Williams, D.M., Niedobitek, G., Young, L.S. 1997. Epstein-Barr virus: biology and disease. Oral Dis. May: 3 Suppl 1:S156-63
4. Weiss, L.M., Movaahed, L.A., Warnke, R.A., Sklar,J. 1989. Detection of Epstein-Barr viral genomes in Reed-Sternberg cells of Hodgkin's disease. N Engl J Med. Feb 23; 320(8): 502-6.
5. MacMahon, E./M., Glass, J.D., Hayward, S.D., Mann, R.B., Becker, P.S., Charade P, McArthur, J.C., Ambinder, R.F. 1991 Epstein-Barr virus in AIDS-related primary central nervous system lymphoma. Lancet. Oct 19; 38(8773):969-73..
6. Weiss, R.A. 1999. Viruses, cancer and AIDS. FEMS Immunol Med Microbiol. Dec; 26(3-4): 27-32.
7. Quintanilla-Martinez. L. Franklin, J.L., Guerrero, I., Krenacs, L., Naresh, K.N., Rama-Rao, C., Bhatia, K., Raffeld, M., Magrath, I.T. 1999. Histological and immunophenotypic profile of nasal NK/T cell lymphomas from Peru: high prevalence of p53 overexpression. Hum Pathol. Jul; 30(7): 849-55.
8. Ott, G., Kirchner, T., Muller-Hermelink, H.K. 1994. Monoclonal Epstein-Barr virus genomes but lack of EBV-related protein expression in different types of gastric carcinoma. Histopathology. Oct; 25(4): 323-9.
9. Borza, C.M., Hutt-Fletcher, L.M.2002. Alternate replication in B cells and epithelial cells switches tropism of Epstein-Barr virus. Nat Med. Jun;8(6): 594-9.
10. Baselga J and Norton L, 2002. Focus on breast cancer. Cancer Cell. 2002 May; 1(4):319-22
11. Labrecque LG, Barnes DM, Fentiman IS, and Griffin BE, 1995. Epstein-Barr virus in epithelial cell tumors: a breast cancer study. Cancer Res. 1995 Jan 1;55(1):39-45.
12. Bonnet M, Guinebretiere JM, Kremmer E, Grunewald V, Benhamou E, Contesso G, and Joab I, 1999. Detection of Epstein-Barr virus in invasive breast cancers. Journal of the National Cancer Instite, Aug. 91(16): 1376-1381.

13. Magrath, I., Bhatia, K., 1999. Breast cancer: a new Epstein-Barr virus-associated disease? J Natl Cancer Inst. Aug 18; 91(16):1349-50.

14. Dadmanesh F, Peterse JL, Sapino A, Fonelli A and Eusebi V, 2001. Lymphoepithelioma-like carcinoma of the breast: lack of evidence of Epstein-Barr virus infection. Histopathology. 2001 Jan;38(1):54-61.

15. McCall SA, Lichy JH, Bijwaard KE, Aguilera NS, Chu WS, and Taubenberger JK. 2001. Epstein-Barr virus detection in ductal carcinoma of the breast. J Natl Cancer Inst. 2001 Jan 17;93(2):148-50.

16. Chu PG, Chang KL, Chen YY, Chen WG, and Weiss LM, 2001. No significant association of Epstein-Barr virus infection with invasive breast carcinoma. Am J Pathol. 2001 Aug;159(2):571-8.

17. Fina F, Romain S, Ouafik LH, Palmari J, Ben Ayed F, Benharkat S, Bonnier P, Spyratos F, Foekens JA, Rose C, Buisson M, Gerard H, Reymond MO, Seigneurin JM and Martin PM, 2001. Frequency and genome load of Epstein-Barr virus in 509 breast cancers from different geographical areas. Br J Cancer. 2001 Mar 23;84(6):783-90.

18. Deshpande CG, Badve S, Kidwai N, and Longnecker R. 2002. Lack of expression of the Epstein-Barr Virus (EBV) gene products, EBERs, EBNA1, LMP1, and LMP2A, in breast cancer cells. Lab Invest. 2002 Sep;82(9):1193-9.

19. Rooney C, Taylor N, Countryman J, Jenson H, Kolman J, and Miller G. 1988. Genome rearrangements activate the Epstein-Barr virus gene whose product disrupts latency. Proc Natl Acad Sci U S A. 1988 Dec;85(24):9801-5.

20. Gan YJ, Razzouk BI, Su T, and Sixbey JW. 2001. A defective, rearranged Epstein-Barr virus genome in EBER-negative and EBER-positive Hodgkin's disease. Am J Pathol. 2002 Mar;160(3):781-6.

21. Takeuchi H, Kobayashi R, Hasegawa M, and Hirai K. 1997. Detection of latent Epstein-Barr virus (EBV) DNA in paraffin sections of nasopharyngeal carcinomas expressing no EBV-encoded small RNAs using in situ PCR. Arch Virol. 1997;142(9):1743-56.

22. Jabs WJ, Hennig H, Kittel M, Pethig K, Smets F, Bucsky P, Kirchner H, Wagner HJ. 2001. Normalized quantification by real-time PCR of Epstein-Barr virus load in patients at risk for posttransplant lymphoproliferative disorders. J Clin Microbiol. 2001 Feb;39(2):564-9.

23. Lichy JH, Zavar M, Tsai MM, O'Leary TJ, Taubenberger JK. 1998. Loss of heterozygosity on chromosome 11p15 during histological progression in microdissected ductal carcinoma of the breast. Am J Pathol. 1998 Jul;153(1):271-8.

24. Yasui Y, Potter JD, Stanford JL, Rossing MA, Winget MD, Bronner M, Daling J. Breast cancer risk and "delayed" primary Epstein-Barr virus infection. Cancer Epidemiol Biomarkers Prev. 2001 Jan;10(1):9-16.

University of Nebraska Medical Center

EPSTEIN-BARR VIRUS AND BREAST CANCER

Guimei Zhou, Kavita Patel, William W. West, Clementina Rao, Labiba Temim, Kishor G Bhatia, Wing C. Chan
Department of Pathology And Microbiology, University Of Nebraska Medical Center, Omaha, NE, 68198-6495
and King Faisal Specialist Hospital And Research Center, Riyadh 11211, Saudi Arabia

Abstract

We collected 100 cases of invasive breast carcinomas, as well as 30 non-neoplastic tissues adjacent to the tumors for studying the evidence of Epstein-Barr virus (EBV) infection. DNA was extracted from these paraffin embedded tissues using EX-WAX DNA extraction kit. Conventional PCR were performed on 30 cases including both tumor and benign tissues, using sets of primers flanking the BWRF1, LMP1, BZLF-1 and EBNA-3C genes. Nineteen of 30 tumor tissues were positive for at least one set of primers (3 cases were positive for all), including 9 cases that were PCR positive for both tumor and benign tissues. However, the correlation among the primer sets used was poor. This may due to the very low copy number of EBV present in tissues and some tissues may have partial deleted EBV genome. Thus, real-time PCR was employed to quantitate the number of EBV genome in tissues. A106-bp region of EBV BamH1K encoding EBNA1 was amplified to detect wild type EBV genome while a 90-bp region that framed the junction of rearranged DNA to detect defective, rearranged EBV genome. A 72-bp DNA sequence of human ApoB gene was used as an internal control. Preliminary data showed that cases, which only have one set of primer positive for PCR, had lower load of the EBV genome.

Association of EBV with breast cancers was also assessed at King Faisal Specialist Hospital, Saudi Arabia with103 breast cancers from two different geographical regions. 40 breast tumors from Kidwai Cancer Center, Bangalore, India and 63 samples from Kuwait Cancer Center. To determine the presence of EBV, amplification of the region flanking BZLF1 gene was carried out. Only 3 of 63 samples from Kuwait were positive for EBV, whereas 16/40 Breast cancer samples from India were EBV positive.

The 40 breast cancer biopsies from India and 30 samples from USA were also assessed for the expression of EBERs using in situ hybridization. All, but 1 sample from the USA, including those that were PCR positive, failed to show expression of EBERs in tumor cells. Two USA samples showed some staining of infiltrated lymphocytes. These data suggest that the incidence and/or the viral load of EBV associated with breast cancers may be geographically variable and there is a discrepancy between the PCR and ISH assays. Part of the discrepancy may be due to the detection of EBV by PCR on infiltrating lymphoid cells. However, the possibility of the presence of partially deleted EBV in tumor cells that failed to express EBERs needs to be explored by further studies.

Figure 1 EBER-1 in situ hybridization in positive control and breast cancer sections. (A) shows strong nuclear staining in a posttransplant lymphoma cells. (B) Case #20 shows nuclear positivity in the infiltrated lymphocytes. (C) Positive staining was detected in focal tumor cells (case # 17R).

Figure 2 PCR amplification of BZLF-1 (A) and LMP-1 (B) genomic DNA on 4 breast cancer samples (lane 1-4 in both A and B). Lane 3 represents a sample which is PCR positive for BZLF-1, but negative for LMP-1. Lane 6 (A) and Lane 5 (B) are Namalwa DNA. Lane 5 (A) and Lane 6 (B) are no template controls. (C) Breast cancer samples amplified with human B-globin DNA to demonstrate good DNA quality.

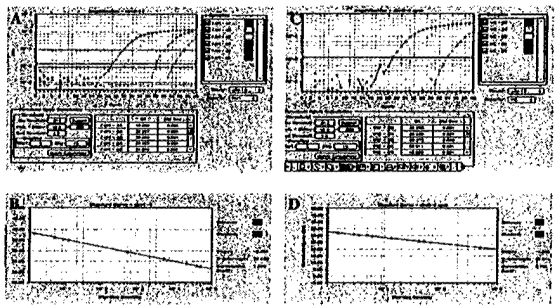

Figure 3. Amplification plot of real-time PCR of EBNA-1. (A) Serially diluted plasmid containing EBV (50,000 to 5 copies per tube) were analyzed for controls. (B) Standard curve generated from the mean value of duplicated examinations. Black circles represent controls and red circles are breast cancer samples and cell lines. (C) and (D) Same samples amplified for human Apo-B DNA to normalize DNA content of each sample. (C) Serially diluted RL-7 DNA (an EBV negative lymphoma cell line) from 500 ng to 0.5 ng were analyzed for controls.

Table 2. PCR PRIMER AND PROBE SEQUENCES

BZLF-1	+5'-AGGCTGTGGAACACCAATG
	-5'-AACCCAGAATCAACAGACTAAC
BWRF-1	+5'-CCATGTAAGCCTGCCTCGAG
	-5'-GCCTTAGATCTGGCTCTTTG
LMP-1	+5'-CGGAAGAGGTTGAAAACAAA
	-5'-GTGGGGGTCGTCATCATCTC
EBNA-3C	+5'-AGAAGGGGAGCGTGTGTTGT
	-5'-GGCTCGTTTTTGACGTCGGC
EBNA-1	+5'-CCGGTGTGTTCGTATATGGAG
	-5'-GGGAGACGACTCAATGGTGTA
EBNA PROBE	FAM-TGCCCTTGCTATTCCACAATGTCGTCTT-TAMRA
APO-B	+5'-TGAAGGTGGAGGACATTCCTCTA
	-5'-CTGGAATTGCGATTTCTGGTAA
APO-B PROBE	VIC-CGAGAATCACCCTGCCAGACTTCCGT-TAMRA

Introduction

Epstein-Barr virus (EBV) is associated with a range of human malignancies, including African Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), classical Hodgkin's disease, post-transplant and AIDS-associated lymphomas, sinonasal NK/T cell lymphoma, and gastric carcinomas.

Breast cancer is the most frequent malignancy and the leading cause of cancer death among women in Western countries. Although the etiology of breast cancer is not completely understood, genetic background and hormonal effects are believed to play important roles in its development. In recent years, researchers have questioned whether EBV may play a role in the development of breast cancer. However, a number of reports on its association with breast cancer have shown conflicting results. This could be related to the different assays employed and also possible geographical variations in the incidence of this infection.

The aims of this study are: 1) To compare if different assays for the detection of EBV provide different endpoints. 2) To compare if there is a geographical variation in the association of EBV with breast cancer samples obtained from the USA, Middle Eastern countries, India and China. 3) To determine if the presence of EBV in these samples is a) restricted to tumor cells and b) if the virus was present prior to the clonal expansion of the tumor cells. 4) To assess the overall expression pattern of the viral genes in EBV positive breast cancer specimens.

Table 1. Summary of EBV-positive breast cancer by PCR and EBER in situ hybridization

	Tumor					Benign				
Case No.	BZLF-1	BWRF-1	LMP-1	EBNA-3C	EBER ISH	BZLF-1	BWRF-1	LMP-1	EBNA-3C	EBER ISH
2	-	+	-	-	-	-	-	+	-	-
3	-	-	-	-	-	-	-	-	-	-
4	+	+	+	+	-	-	-	-	-	-
6	+	-	-	+	-	-	-	-	-	-
8	+	-	-	-	-	+	-	-	-	-
9	+	-	-	-	-	NA	NA	NA	NA	NA
12	+	+	-	-	+L	+	-	-	-	-
13	+	-	-	-	-	+	-	-	-	-
14	+	-	-	-	-	-	-	-	-	-
15	+	-	-	-	-	+	-	-	-	-
17R	+	+	-	-	+T	+	-	-	-	-
17L	-	-	-	-	-	+	-	-	-	-
18	+	-	-	-	-	-	-	-	-	-
19	+	-	-	-	-	-	-	-	-	-
20	+	+	-	+	+L	+	-	-	-	-
22	+	-	-	-	-	-	-	-	-	-
23	+	-	-	-	-	+	-	-	-	-
25	+	-	-	-	-	-	-	-	-	-
27	+	-	-	-	-	NA	NA	NA	NA	NA
29	+	-	-	-	-	-	-	-	-	-

NA- not available; +L – positive in lymphocytes; +T – positive in tumor cells

Conclusions

EBV does present in a small subset of breast carcinomas, however, the high PCR positivity is largely an artifact of latently infected lymphocytes.

The incidence and/or the viral load of EBV associated with breast cancers may be geographically variable.

There is a discrepancy between the PCR and ISH assays.

Acknowledgment

The U.S. Army Medical Research Materiel Command under DAMD17-01-1-0340 (to WCC) supported this work.

Drs. Wing C. Chan & Guimei Zhou
986495 Nebraska Medical Center
Omaha, NE 68198-6495
jchan@unmc.edu & gzhou@unmc.edu